REMARKS

Claims 1-45 have been canceled. New claims 46-89 have been added to solely address the 35 U.S.C. § 112 rejections and not to avoid any prior art. Claims 46-89 are pending in the present application.

It is respectfully submitted that the present amendment presents no new issues or new matter and places this case in condition for allowance. Reconsideration of the application in view of the above amendments and the following remarks is requested.

I.   The Restriction Requirement

The Office Action made a restriction requirement between the following groups:

Group I: claims 1-22, 27, and 44-45, drawn to polypeptides, compositions comprising said polypeptides, and a method for producing said polypeptides;

Group II: claims 23-26 and 28-29, drawn to nucleic acid sequences, constructs, vectors, host cells, and methods for recombinantly producing said polypeptide;

Group III: claims 30-31, drawn to a cell mutant and a method for producing said cell mutant;

Group IV: claim 32, drawn to a method for producing a heterologous polypeptide;

Group V: claims 33-39, drawn to a protein hydrolysate and methods for producing said protein hydrolysates from a proteinaceous material; and

Group VI: claims 40-43, drawn to a protein hydrolysate enriched in free glutamic acid and/or peptide bound glutamic acid residues, and methods for obtaining said protein hydrolysate.

As provided therein, Applicants provisionally elected with traverse the claims of Group I. Applicants confirm this election. Applicants reserve the right to file continuing applications directed to the non-elected subject matter.

II.   The Rejection of Claims 1-22, 27, and 44-45 under 35 U.S.C. § 112, Second Paragraph

Claims 1-22, 27, and 44-45 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite on several grounds.

Ground 1: The Office Action states that claim 1 and dependent claims thereof are indefinite because the terms "medium stringency" and "high stringency" in the specification on page 11 do not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. This rejection is respectfully traversed.

Applicants respectfully point out that prehybridization and hybridization conditions are described on page 5, lines 26-31 and wash conditions are described on page 6, lines 11-16, of the specification. Using these conditions, the skilled artisan could readily determine whether a polypeptide encoded by a nucleic acid sequence is within the scope of the claims.

Ground 2: The Office Action states that claims 1-22, 27, and 44-45 are indefinite because the term "% identity" is not defined with respect to the parameters required to use the Clustal method. This rejection is respectfully traversed.

Applicants respectfully point out that % identity is measured by the Clustal method using the parameters described on page 4, lines 18-20, of the specification. The Clustal method is well known in the art for determining sequence alignments as described by Higgins *et al.* in *CABIOS* 5: 151-153 (1989).

Ground 3: The Office Action states that claims 19-21 are indefinite because the term "relative to initial activity" is not defined with respect to a standard for ascertaining the requisite degree of the metes and bounds of the term. This rejection is respectfully traversed.

Applicants respectfully point out that the term "relative to initial activity" simply refers to the activity of the enzyme prior to incubation for 20 minutes at 60°C and pH 7.5.

Ground 4: The Office Action states that in claim 44 the term "flavor-improving composition" is not defined with respect to a standard for ascertaining the requisite degree of the metes and bounds of the term. This rejection is respectfully traversed.

The term "flavor-improving composition" would be understandable by the skilled artisan to mean that treatment of a proteinaceous substrate with the composition would produce a flavor which is improved relative to the untreated substrate. However, to advance prosecution, the newly presented claims do not use the term "flavor-improving composition".

For the foregoing reasons, Applicants submit that the new claims overcome the rejections under 35 U.S.C. § 112. Applicants respectfully request reconsideration and withdrawal of the rejection.

III. The Rejection of Claims 1, 7, 11-18, and 27 under 35 U.S.C. § 102

Claims 1, 7, 11-18, and 27 stand rejected under 35 U.S.C. § 102(e) as being anticipated by Holm *et al.* (U.S. Patent No. 5,821,104). The Office Action states:

> Holm *et al.* disclose an isolated polypeptide having aminopeptidase activity wherein said polypeptide is encoded by a nucleic acid sequence which hybridizes with the nucleic acid sequence or a subsequence of SEQ ID NO:1 or its complementary strand (as stated in instant claim 1) (see entire reference) and wherein said polypeptide is obtained from an *Aspergillus oryzae* strain. Holm *et al.* also disclose a polypeptide comprising a fragment of the sequence of instant SEQ ID NO:2 and a method for producing said polypeptide comprising recovering said polypeptide from said *Aspergillus* strain (see entire reference).

This rejection is respectfully traversed.

Holm *et al.* disclose a tripeptidyl aminopeptidase, a DNA construct encoding the tripeptridyl aminopeptidase, a method of producing the tripeptidyl aminopeptidase, and methods of reducing the tripeptidyl aminopeptidase production in cells in which tripeptidyl aminopeptidase activity is undesirable. The tripeptidyl aminopeptidase sequentially removes intact tripeptides from the N-terminus of a peptide, polypeptide, or protein. (See page 1, lines 20-22 of the Holm *et al.* reference).

However, Holm *et al.* do not disclose an aminopeptidase which sequentially removes one amino acid residue at a time from the N-terminus of a peptide, polypeptide, or protein, as claimed herein.

An aminopeptidase is not a tripeptidyl aminopeptidase. Applicants provide a copy of an article by Taylor, 1993, *The FASEB Journal* 7: 290-298, which defines an aminopeptidase (see page 290, column 2, first complete paragraph).

For the foregoing reasons, Applicants submit that this rejection under 35 U.S.C. § 102 has been overcome. Applicants respectfully request reconsideration and withdrawal of the rejection.

IV. The Rejection of Claims 1, 7, 11-18, and 27 under 35 U.S.C. § 102

Claims 1, 7, 11-18, and 27 stand rejected under 35 U.S.C. § 102(e) as being anticipated by Kauppinen *et al.* (WO 96/285420). The Office Action states:

Kauppinen *et al.* disclose an isolated polypeptide having aminopeptidase activity wherein said polypeptide is encoded by a nucleic acid sequence which hybridizes with the nucleic acid sequence or a subsequence of SEQ ID NO:1 or its complementary strand (as stated in the instant claim 1) (see entire reference) and wherein said polypeptide is obtained from an *Aspergillus oryzae* strain (see entire reference, for example pages 4 and 6). Kauppinen *et al.* also disclose a polypeptide comprising a fragment of the sequence of instant SEQ ID NO:2. A method for producing said polypeptide comprising recovering said polypeptide from said *Aspergillus* strain (see entire reference) and compositions comprising said aminopeptidase as recited in the instant claims 44-45 (see entire reference).

This rejection is respectfully traversed.

Kauppinen *et al.* disclose an *Aspergillus oryzae* aminopeptidase having a molecular weight of 35 kDa.
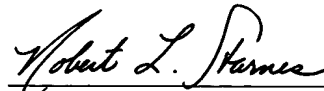
Applicants submit herewith a Declaration of Dr. Alexander Blinkovsky. The Declaration provides that the amino acid sequence of the Kauppinen aminopeptidase is 13.5% identical to the aminopeptidase of SEQ ID NO. 2. This low degree of identity between the amino acid sequences of the two aminopeptidases indicates that the corresponding genes, and subsequences of the genes which encode polypeptides having aminopeptidase activity, would not hybridize under medium stringency conditions as defined by prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and 35% formamide for medium and wash conditions of three times each for 15 minutes using 2 x SSC, 0.2% SDS at 55°C, following standard Southern blotting procedures. A subsequence is a nucleic acid sequence where one or more nucleotides have been deleted from the 5' end and/or 3' end of the nucleic acid sequence, wherein the subsequence encodes a polypeptide fragment which has aminopeptidase activity. The polypeptide fragment preferably contains at least 330 amino acid residues. (See page 4, lines 30-31, and page 9, lines 25-26, of the specification).

For the foregoing reasons, Applicants submit that this rejection under 35 U.S.C. § 102 has been overcome. Applicants respectfully request reconsideration and withdrawal of the rejection.

V. Conclusion

In view of the above, it is respectfully submitted that all claims are in condition for allowance. Early action to that end is respectfully requested. The Examiner is hereby invited to contact the undersigned by telephone if there are any questions concerning this amendment or application.

Respectfully submitted,

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Aminopeptidases: structure and function

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ABSTRACT   Aminopeptidases catalyze the cleavage of amino acids from the amino terminus of protein or peptide substrates. They are widely distributed throughout the animal and plant kingdoms and are found in many subcellular organelles, in cytoplasm, and as membrane components. Several aminopeptidases perform essential cellular functions. Many, but not all, of these peptidases are zinc metalloenzymes and are inhibited by the transition-state analog bestatin. Some are monomeric, and others are assemblies of relatively high mass (50 kDa) subunits. cDNA sequences are available for several aminopeptidases, and a 3-dimensional structure is available for the bovine lens enzyme. Crystallographic, electron micrographic, NMR, and photoaffinity labeling studies indicate that lens leucine aminopeptidase protomers are bilobal and that bestatin and substrates are bound in an active site, which is found in the larger lobe on each protomer. Zn^{2+} is involved in substrate liganding in most aminopeptidases. There is no evidence of an acyl-enzyme intermediate in hydrolysis. Amino acid sequences determined directly or deduced from cDNAs indicate some amino acid sequence homologies in organisms as diverse as *Escherichia coli* and mammals, particularly in catalytically important residues or in residues involved in metal ion binding.——Taylor, A. Aminopeptidases: structure and function. *FASEB J.* 7: 290–298; 1993.

Key Words: aminopeptidase · leucine aminopeptidase · bestatin · Zn^{2+}

AMINOPEPTIDASES CATALYZE THE CLEAVAGE of amino acids from the amino terminus of many proteins. They were some of the earliest proteases discovered (1, 2). Shortly afterward, work related to composition and structure began in earnest. It was primarily in the last two decades, however, that substantial information about the structure, mechanism of action, biological function, and regulation of expression was significantly elucidated. Recent progress regarding structural aspects and the mechanism of action of mammalian aminopeptidases is reviewed here. Another summary that stresses function of—and regulation of expression of—these enzymes is in preparation (A. Taylor, *TIBS*, in press). Many hundreds of citations exist regarding aminopeptidases. Due to space restrictions, reference is made to review articles in which a substantial amount of the earlier progress is summarized (3–10) and/or to selected newer work. I apologize in advance to those authors whose good work is not directly cited and to those whose work I was forced to delete.

CLASSIFICATION AND NOMENCLATURE

Many aminopeptidase activities have been described, and they appear to be widely distributed in the plant and animal

kingdoms. They are essential for protein maturation (11), degradation of nonhormonal (12) and hormonal peptides, and possibly determination of protein stability (13), etc. Many disease states are associated with impaired proteolytic function (14).

Aminopeptidases are classified:

1) By the number of amino acids cleaved from the NH_2 terminus of substrates. Enzymes that sequentially remove the NH_2-terminal amino acid from protein and peptide substrates are called aminopeptidases. Aminodipeptidases, or diaminopeptidases, remove intact NH_2-terminal dipeptides. Aminotripeptidases catalyze the hydrolysis of NH_2-terminal tripeptides (10, 15). These are given the numbers E.C. 3.4.11–3.4.13.

2) With respect to the relative efficiency with which residues are removed. Leucine aminopeptidases (LAP)[1] remove most effectively Leu and other hydrophobic residues from peptide substrate analogs, although bonds to many other residues are cleaved. Arginine-, methionyl-, aspartyl-, alanyl-, glutamyl-, prolyl-, and cystinyl-aminopeptidases have also been described. In most instances, the kinetic tests used for identification were done with amino analogs (i.e., amino acyl-*p*-nitroanilides or -*β*-naphthylamides) as substrates, and these are rarely hydrolyzed at rates comparable to rates of hydrolysis of peptides that bear the same amino-terminal residue. In addition, metal ion content in many enzymes have rarely been held constant. Each of these influences relative activities, making classification according to the residue cleaved a nettlesome chore (16, 17). For example, lens LAP in the $Zn^{2+}Mg^{2+}$ form (see metal content below) shows a specific activity for a typical physiological substrate, LeuGlyGly, of 804 μmol · min^{-1} · mg^{-1} and a K_m of only 1 mM. In contrast, for the widely used substrate leucyl-*p*-nitroanilide (LpNA), the specific activity is 3.9 μmol · min^{-1} · mg^{-1}; yet the K_m is similar (approximately 3 mM) to that for the peptide. In comparison, AlaGlyGly is also rapidly hydrolyzed (specific activity 604 μmol · min^{-1} · mg^{-1}), although the K_m is 50 times as great (16). Analogous information regarding hog kidney (hk) LAP was obtained by Lin and Van Wart (18). The difficulty in utilizing substrates for characterization is corroborated by Patterson's (19) observation of different recovery times for bestatin-inhibited aminodipeptidase utilizing different substrates.

3) With respect to location of the aminopeptidase. Some peptidases are secreted (20), but most are not. There are

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soluble and microsomal enzymes, membrane-bound and cytosolic enzymes. Other aminopeptidases are found in organelles, such as lysosomes, nuclei, and mitochondria. In some instances, membrane-bound and methionine aminopeptidases share the same name — aminopeptidase M. It is not uncommon for structurally unrelated enzymes to have the same name and to be found in different locations within one organism.

4) By susceptibility to inhibition by bestatin, a transition-state analog of the dipeptide substrate PheLeu (**Table 1**).

5) According to metal ion content and/or the residues that bind the metal to the enzyme (21; Wallner et al., unpublished results) (see metal content below).

6) According to the pH at which maximal activity is observed. There are acidic, basic, and "neutral" peptidases.

Obviously, each of these nomenclature or classification systems serve to identify the protease with respect to a topic of interest, and they are not mutually exclusive. Given the labyrinth of classifications, it is not surprising that in recent years several enzymes previously thought to be distinct were shown to be identical. For example, hkLAP and hog intestinal prolyl aminopeptidase are indistinguishable (22). The same pertains to rat kidney and brain prolyl aminopeptidase and hog kidney LAP (23). The *Escherichia coli xerB* gene product, *E. coli* aminopeptidase I, now called aminopeptidase A, and *Saccharomyces typhimurium* aminopeptidase appear to be the same (24), as are aminopeptidases N and M (E.C. 3.4.11.2) (10). Perhaps it would be advantageous to assay an aminopeptidase with many peptide substrates before choosing a name.

STRUCTURE

Despite the wide variety of aminopeptidases isolated, significant amino acid sequence information is published for only about six of these enzymes (**Table 2**). Among those aminopeptidases for which sequence information is available, only limited homologies have been observed to date. This may be surprising as these enzymes are ubiquitous, and kinetic features (and in some cases metal binding ligands) show considerable similarity (Table 1, see below). Perhaps one of the most intriguing relationships is the observation of significant levels of homology between mammalian LAP from bovine lens (bl) or bovine kidney (bk) and *E. coli* aminopeptidase A (24; B. Wallner et al., unpublished results). Currently, lens LAP is the only aminopeptidase for which a three-dimensional structure is available (25). This structure is described first. Comparisons with other aminopeptidases follow.

Although crystals of LAP were obtained more than two decades ago, initial information regarding the structure of the lens LAP molecule and the organization of the blLAP hexamer were obtained by electron microscopy of single molecules (**Fig. 1A**) and crystal thin sections (26, 27). The shape of the blLAP protomer was described as bilobal (Fig.

TABLE 1. *Dissociation constants for Bestatin and Aminopeptidases*

Enzyme	Type of inhibition	K_i^* (M) [Substrate][a]	K_i (M)	Me^{2+}	Reference
Mouse ascites tumor dipeptidase	Competitive, slow	2.7×10^{-9} [dipeptides]		Mg^{2+}	(19)
Aminopeptidase M	Competitive, slow	4.1×10^{-6} [LpNA]	7×10^{-6}	$Zn^{2+}Mg^{2+}$	(44) (41)
	Not slow	1.4×10^{-6} [LpNA]			(32)
Hog kidney cytosolic leucine aminopeptidase	Competitive, slow	2×10^{-8} [LpNA]		$Zn^{2+}Mg^{2+}$	(44)
	Competitive, slow	2×10^{-8} [LpNA]		$Zn^{2+}Mg^{2+}$	(41)
	Competitive, slow	5.8×10^{-10} [LpNA]		$Zn^{2+}Mn^{2+}$	(32)
	Competitive, slow[b]	2×10^{-8} [LeuGlyGly]	$\sim 10^{-8}$	$Zn^{2+}Zn^{2+}$	
		2×10^{-8} [LβNA]		$Zn^{2+}Zn^{2+}$	(31)
Aeromonas	Competitive, slow	1.8×10^{-8} [LpNA]			(32)
Bovine lens leucine aminopeptidase	Competitive,[c] slow	1.3×10^{-9} [LeuGlyGly]	1.1×10^{-7}	Zn^{2+},Zn^{2+}	
Bovine lens leucine aminopeptidase (azidobestatin)[d]	Competitive, slow	4×10^{-9} [LeuGlyGly]	10^{-8}	Zn^{2+},Zn^{2+}	(40)
Aminopeptidase B	Competitive, slow	6×10^{-8} [LβNA]			(54)

[a]The K_m for all the substrates is approximately mM. [b]Hakamian and Taylor, unpublished results. [c]Taylor et al., Biochemistry, in press. [d]Note the inhibitor used was azidobestatin.

TABLE 2. *Aminopeptidases for which structural and compositional data are available*

Names and source[a]	Subunit mass (kDa) (no. of subunits)	Total mass (kDa)	Metal ion residues used to ligand metal ion	Unique gene?	Primary sequence known or deduced?	Preprotein size (kDa) (size of NH$_2$-terminal extension, kDa)	Reference
Rat kidney membrane zinc aminopeptidase	140 (1)	140	Zn^{2+}	Yes	Yes		(50)
Rat kidney membrane aminopeptidase M	110 966 residues		Zn^{2+} His His Glu		Yes		(21)
Liver arginine aminopeptidase	90 (1)	90					(43)
Muscle aminopeptidase N[b]	51	390					(55)
Beef/hog[c] lens/kidney leucine aminopeptidase	53 (bl and bk) 487 residues (6)	318	2 Zn^{2+} per subunit Asp Glu Lys	Yes	Yes	55.6 (bl and bk) 514 residues (26 residues)	(B. Wallner et al., manuscript; submitted; 25, 37, 39)
E. coli aminopeptidase A *XerB* gene product	55.3 503 residues						(24)
Yeast, yscl, APEI/ LAP4	44.8 (12) subunits 469 residues	640		Yes		57.0 514 residues	(56)
	51.7					57.2 514	(57)
Yeast aminopeptidase A peptidase 4 gene product	44		2 Zn^{2+}	Yes			(58)
Aeromonas LAP	30, 32 thermostabile (1)		2 Zn^{2+}		Yes	43 thermolabile (~13 kDa)	(49)
			1 Zn^{2+} required, can bind another Zn^{2+}				(38)
Mouse ascites dipeptidase			1 Zn^{2+}				(19)
Salmonella methionine aminopeptidase	34		no metal				(59)
E. coli methionine aminopeptidase	29.3 (1) 264 residues	29.3			Yes		(52)
Yeast methionine aminopeptidase	43.3 377 residues		Co^{2+}		Yes	387 residues (10 residues)	(51)
		~42 ~34	Co^{2+}			42 (~10 kDa)	
Ochrobactrum anthropi SCRC C1-38 D-amino acid aminopeptidase	59		No metal				(47)

[a]In some cases, similar enzyme names are used, but it is not clear that the enzymes are related. Thus they are listed separately. Where clear associations have been noted, the enzymes are grouped together. [b]Not inhibited by bestatin. [c]Bovine lens and hk LAP may differ in that activity is detected in hkLAP in which only 1 equivalent of Zn^{2+} is bound per subunit.
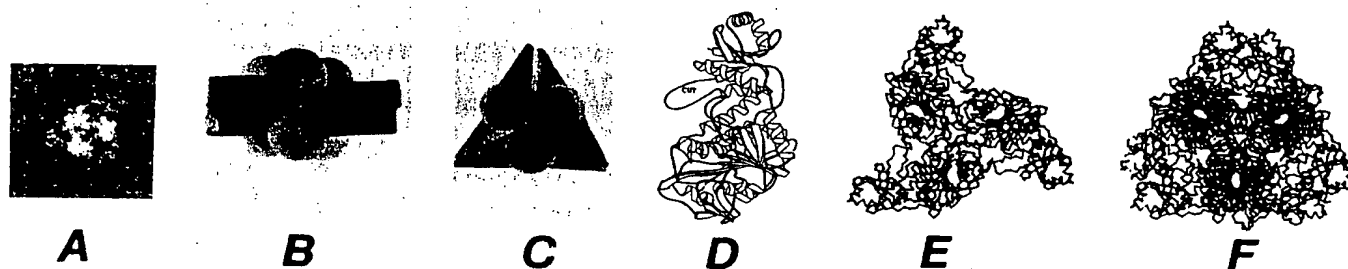
Figure 1. Structure of bovine lens LAP. *A)* Enhanced electron micrograph of a single blLAP molecule embedded in sodium phosphotungstate. *B, C)* Photographs of LAP hexamer model along the twofold (*B*) and threefold (*C*) axis of symmetry. The spherical portion of the subunit contains about two-thirds of the protein and the smaller appendage (shown here as a wedge) contains about one-third of the protein. *D)* Schematic ribbon drawing of the monomer based on crystallographic data (25). The upper portion contains the minor lobe (⅓) and the lower portion contains the major lobe (⅔). *E, F)* Tracing of the carbon backbone of the blLAP trimer (*E*) and hexamer (*F*) as viewed down the threefold axis.

1*B–D*), the distribution of protein being two-thirds, one-third between the larger and smaller lobes, respectively (26). Hexamers appeared as two concentric triangles, the smaller being offset from the larger "less dense" triangle by 60°. The shape of the protomer and the arrangement of protomers within the hexamer (a dimer of trimers) were corroborated by crystalographic analysis (25). Centers of each of the two blLAP hexamers contained in the lens LAP unit cell were at (⅓, ⅔, ¼) and (⅔, ⅓, ¾) along a, b, and c axes of the unit cell (26). The space group for lens LAP is P6₃22 for crystals grown in ammonium sulfate, methyl pentanediol, or lithium sulfate (25, 27; A. Taylor, unpublished results). The maximum length of the blLAP molecule along the threefold axis of symmetry is 90 Å. The edge of the triangle is ~ 113 Å (25, 26). The shape of protomers and their arrangement within the hkLAP hexamer are indistinguishable from that observed in blLAP, although hkLAP crystalized in the space group P2₁2₁2₁ (28). Cuypers et al. (29) provided the first amino acid sequence of blLAP. Trypsinization of the enzyme resulted in the formation of only two polypeptides, one with a mass of 37 kDa (residues 138-487) and the other with mass of 18 kDa (residues 1-137) (Fig. 1*D*). A unique cleavage between residues 322 and 323 by hydroxylamine results in similarly sized but different polypeptides (1-322 and 323-487) from those obtained with trypsin. The availability of a deduced amino acid sequence for a purportedly identical aminopeptidase isolated from bovine kidney (see homology below) allowed some refinements of the amino acid sequence determined by protein sequencing techniques. These include recognition of an eight-residue fragment near the carboxyl terminus and several single amino acid substitutions or additions (B. Wallner et al., unpublished results). This enhanced sequence information was essential for solution and refinement of the structure to 2.3 Å resolution, as well as for comparison of the blLAP and *E. coli* aminopeptidase A structures (Fig. 2) (24, 25; B. Wallner et al., unpublished results).

The NH₂-terminal 150 residues fold to give a β-sheet sandwiched between four α-helices. The NH₂ terminus itself occurs as the middle strand of the sheet (25). It is this portion of the molecule that comprises the smaller lobe of each protomer. There is a long loop connecting an α-helix and the fifth strand of the β-sheet. This long loop contains the peptide bond between residues Arg-137 and Lys-138, where trypsin cleaves each protomer (see CUT, Fig. 1*D*) within the hexameric enzyme but leaves the hexamer intact, fully active (30), and crystalizable (F. H. Carpenter, unpublished results). The effective stabilization of hexamers in the native and, presumably, in the clipped enzymes is achieved via an extensive network of hydrogen bonds and van der Waals' interactions (25).

Mode of inhibitor binding

Before proposing a mechanism of inhibitor binding or a mechanism of peptide hydrolysis, it was necessary to determine the number of active sites per hexamer, locate the inhibitor and/or substrate binding sites, and reconcile previously observed metal-ion/substrate interactions.

The discovery of a relatively tight-binding inhibitor of LAP, bestatin [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine],
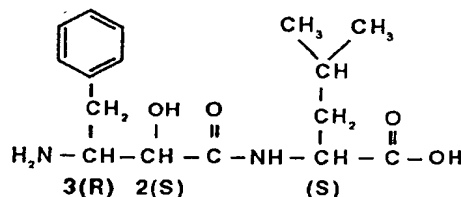
heralded new opportunities to identify the active site (31). Direct measures of LAP saturated with bestatin (A. Taylor et al., in press)[2] and inactivation assays indicated that each subunit of blLAP binds a bestatin. This is consistent with the identical structure of protomers in bestatin-inhibited LAP crystals (25). Similar results were obtained with p-azidobestatin (C. Z. Peltier and A. Taylor, unpublished results). These data are inconsistent with inactivation assays, which indicate that binding of only one bestatin is sufficient to substantially inactivate hk LAP (32).

Conversion of bestatin to [³H]p-azidobestatin resulted in an effective photoaffinity label for blLAP. After equilibrating [³H]p-azidobestatin and LAP, activation of the label by exposure of the mixture to UV light, and digestion with trypsin or hydroxylamine, the radioactivity was contained within the large tryptic fragment and the small hydroxylamine polypeptide. This demonstrated that the bestatin binding site and presumably the active site are within the carboxyl third of the subunit. This was corroborated by NMR (see below) and crystalographic data, which indicated that the phenylalanyl side chain of bestatin is bound to a hydrophobic pocket (presumably S₁, according to Schechter and Berger [33]) comprised of Met-270, Thr-359, Gly-362, Ala-451, and Met-454 and that the leucyl side chain binds to another hydrophobic cleft (S₁') including residues Asn-330, Ala-333, and Ile-421 (**Fig. 3A**). The backbone of the inhibitor is stabilized in the enzyme by hydrogen bonds involving Lys-262, Asp-273, and Leu-360. Presumably, the substrate LeuGlyGly would be bound in S₁,S₁',S₂' (Figs. 3*B* and 3*C*).

```
BLLAP    TKGLVLGIYSKEKEEDEPQFTSAGENFNKLVSGKLREILNISGPPLKAGK         50
         |  |*|*||        |   |||  |*|||*|| * | |* .|   * *
pepA     SACIVVGVF------EPRRLSPIAEQLDKISDGYISALLRRGELEGKPGQ         58
STRUC    SSSSSS    DDD        HHHHHHHH   HHHHHH         SS


BLLAP    TRTFYGLHEDFPSVVVVGLGKKTAGIDEQENWHEGKENIRAAVAAGCRQI        100
         *  |   |     |||  | *    |** |       ||  |  |*||
pepA     TLLLHHVPNVLSERILLIGCGKERELDERQYKQVIQKTINTLNDTGSMEA        108
STRUC    SSSSS.       SSSSSSS     SS      SSHHHHHHHHHHHHHHHHHH


BLLAP    QDLEIPSVEVDPCGDAQAAAEGAVLGLYEYDDLKQKRKVVVSAKLHGSED        150
          |*|       |      *|* *  |** |*|** *    * ||    |
pepA     VCFELHVKGRNNYWKVRQAVETAKETLYSFDQLKRKMVFNVPTRRELTSG        170
         LT                             TNKSEPRRPL
STRUC    HH    SSSSS     HHHHHHHHHH                   SSSSS


BLLAP    QEAWQRGVLFASGQNLARRLMETPANEMTPTKFAEIVEENLKSASIKTDV        200
         | *|*|*| |*|*   *| * | * *    ||* || * *  ||
pepA     ERAIQHGLAIAAGIKAAKDLGNMPPNICNAAYLASQARQLADSYSKNVIT        220
STRUC    HHHHHHHHHHHHHHHHHHHHHHHH    HHHHHHHHHHHHHHH   SSS


                                                       Z
BLLAP    FIRPKSWIEEQEMGSFLSVAKGSEEPPVFLEIHYKGSPNASEPPLVFVGK        250
         |      | *  * *|*|*| **|| || * *** **** *|*|***
pepA     RVIGEQQMKELGMHSYLAVGQGSQNESLMSVIEYKG--NASERPIVLVGK        270
STRUC    SSS HHHHH    HHHHHH      SSSSSSS         SSSSS


         Z   . B       B Z       .         .         .
BLLAP    GITFDSGGISIKAAANMDLMRADMGGAATICSAIVSAAKLDLPINIVGLA        300
         *|********** |  ** *| **|***|| ||| |* *|****||*|
pepA     GLTFDSGGISIKPSEGMDEMKYDMCGAAAVYGVMRMVAELQLPINVIGVL        320
STRUC    SSSSS             HHHHHHHHHHHHHH       SSSSSS


                                     B  ZBZ      .         .
BLLAP    PLCENMPSGKANKPGDVVRARNGKTIQVDNTDAEGRLILADALCYAHTFN        350
         *****|*|* |****|  | * *||* **********|*|*|*|   *|
pepA     AGCENMPGGRAYRPGDVLTTMSGQTVEVLNTDAEGRLVLCDVLTYVERFE        370
STRUC    SSSSS        SSSSSS SSSSSS     HHHHHHHHHHHHHH


         BB B        .        .         .
BLLAP    PKVIINAATLTGAMDIALGSGATGVFTNSSWLWNKLFEASIETGDRVWRM        400
         * ||*||****** ****  **|||* *  * ** ||***|**|
pepA     PEAVIDVATLTGACVIALGHHITGLMANHNPLAHELIAASEQSGDRAWRL        420
STRUC    SSSSS   HHHHH      SSSS  HHHHHHHHHHHH     SSS


                        .B       .         .         .
BLLAP    PLFEHYTRQVIDCQLADVNNIGKYRSAGACTAAAFLKEFVTHPKWAHLDI        450
         **  |  *   * |||||**| *** * |** **||** *|  ******
pepA     PLGDEYQEQ-LESNFADMANIGG-RPGGAITAGCFLSRFTRKYNWAHLDI        469
STRUC    HHHHH    SSSS      HHHHHHHH     SSSSS


         B  B      .         .         .
BLLAP    AGVMTNKDEVPYLRKGMAGRPTRTLIEFLFRFSQDSA                      487
         **|      |      ** |***| |*||**|   |   |
pepA     AGTAWRSGKA----KGATGRPVALLAQFLLNRAGFNGEE                     503
STRUC    SS         SS   HHHHHHHHHHHH DDD
```

Figure 2. Comparison of the amino acid sequences of bovine lens and *E. coli* aminopeptidase A (*pepA*) showing the secondary structure of blLAP. (*) identity, (|) similarity; structure codes: S, β-strand; H, α-helix; D, disordered loop or terminus. Z indicates zinc ion binding residues. B indicates residues involved in binding bestatin (adapted from ref 25). Residues of *pepA* written below the line at 111-112 and 150-159 are found in the *pepA* sequence between 111 and 112, and 154 and 155, respectively, and were removed to enhance alignment.
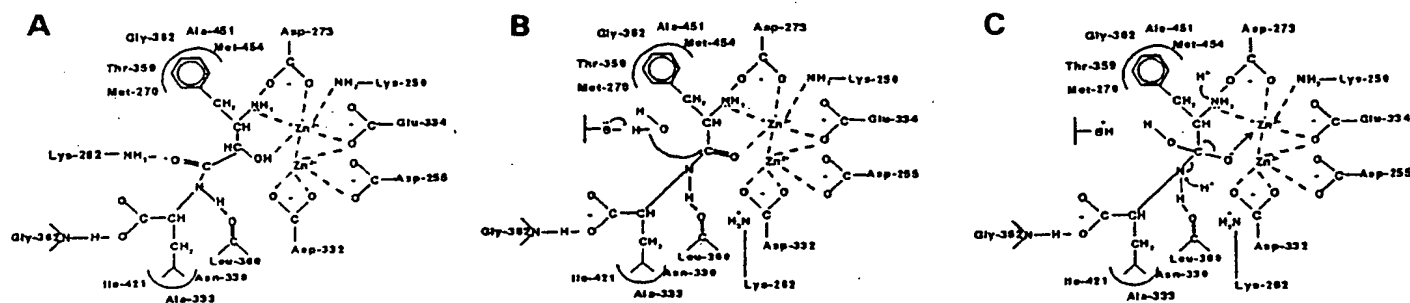
Figure 3. Proposed mode of bestatin binding (*A*) and peptide hydrolysis (*B, C*). See text for details.

Metal ions and mechanism of action

Carpenter and Vahl (34) showed that native lens LAP protomers bind two zinc ions, each with different avidity, and that blLAP is active only when both metal-ion binding sites per subunit are occupied. Since then, most, but not all, aminopeptidases have been identified as metalloenzymes (T-1 and T-2).

Mn²⁺, Mg²⁺, and Co²⁺ can readily be exchanged for the zinc ion at the more readily exchanged site. The apparent dissociation constants for blLAP and Mg²⁺ and Zn²⁺ or Mn²⁺ and Zn²⁺ are 150 and 35, respectively, at pH 8.5. NMR studies showed that the carbonyl oxygen of substrate analogs (i.e., leucyl-*o*-sulfonic acid) is within one hydration radius of Mn²⁺ in blLAP, in which the more readily exchangeable Zn²⁺ has been replaced by Mn²⁺ (35). Thus, the scissile carbonyl oxygen of substrates (Fig. 3*B*), or C-2 OH of substrates (required for tighter binding than peptides) (Fig. 3*A*), is depicted coordinated to the zinc ion located in the readily exchangeable cation-binding site in the native enzyme. The α-amino group (probably unprotonated under physiological conditions) of bestatin and substrates also appears to be coordinated to this ion. Similar proposals regarding binding of substrates and inhibitors to leucine aminopeptidases, based on kinetic data, were made earlier (31, 36). Presumably, the Zn²⁺ occupies this site in native blLAP, which is liganded by Lys-250, Asp-273, and Glu-334, and possibly Arg 336 (25).

Co²⁺ can also replace Zn²⁺ at the less readily exchanged site (37). This ion is liganded by residues Asp-255, Asp-332, and Glu-334. Close proximity (2.9 Å) of the two ions was observed in blLAP as had been predicted earlier for *Aeromonas* aminopeptidase (32). In blLAP, substitution at both metal-ion sites affects both K_m and k_{cat}. This suggests a complex interaction between the two metal ions with each other and/or with substrate. These effects may also be associated with the slight shift in metal-ion positions upon binding inhibitor, and presumably substrate. The short distance between the two zinc ions has been noted in other proteins (25), but there is no other example reported in which Lys is used to coordinate the metal ion.

The monomeric native *Aeromonas* aminopeptidase also binds two equivalents of zinc per subunit but only one appears to be required for activity (38). The addition of Mn²⁺ or Mg²⁺ does not affect the activities measured. Perturbation of the cobalt spectrum upon binding inhibitors suggests that substrates or inhibitors are within the immediate vicinity of metal ions in *Aeromonas* aminopeptidase as well (32). Although highly homologous with blLAP, hk LAP appears to bind tightly only one equivalent of zinc, but like blLAP, it is activated significantly upon binding of Mg²⁺ and Mn²⁺

ions (39). It is interesting that in all three enzymes, kinetic and/or structural investigations indicate the possibility of an interaction between the metal ions.

The consistency of data that each subunit can bind a bestatin allowed use of NMR and X-ray diffraction information to infer a mechanism of hydrolysis of peptide substrates (Figs. 3*B* and 3*C*). The more readily exchanged zinc ion would appear to provide electrophilic interaction to the substrate carbonyl to initiate the reaction and/or stabilize the tetrahedral intermediate. The reaction could be driven by a proton provided by Lys-262 and/or H₂O (25). Hydrolysis also involves nucleophilic attack at the carbon of the scissile carbonyl. There are no enzyme nucleophiles in the area of the scissile peptide bond. This is consistent with an inability to inactivate the enzyme using a variety of affinity labels, which required attack by an enzyme-bound nucleophile for covalent attachment (16, 40). Accordingly, general base catalysis is suggested for the mechanism of hydrolysis of peptides (31). The most plausible nucleophile would appear to be H₂O or OH⁻ stabilized and/or protonated by zinc. Patterson (19) suggested that slow removal or displacement of an OH⁻ from a "deep pocket" in an aminodipeptidase may be related to the slow binding of bestatin and that the C2-OH may be the functional group responsible for that displacement. Upon hydrolysis, an increase in pK of the α-NH₂ to more than 9 would result in protonation and release from the active site.

These data also allow rationalization of several unexpected observations. Thioamide derivatives did not show enhanced binding to hkLAP (41). The LAP used for that work was in the Zn²⁺Mg²⁺ form. Assuming that hkLAP and blLAP have the same metal ion distribution, it would appear that Mg²⁺, and not Zn²⁺, was in the readily exchanged site. This might explain the unexpected decrease in apparent affinity found for thioamides in the hkLAP, as Mg²⁺ does not have the same binding affinity for sulfur-containing compounds as Zn²⁺. This rationale would not appear to explain the lack of binding enhancement in binding thiobestatin and brain aminopeptidase under conditions where zinc might be expected to be retained in the enzyme (42). Alternate functions for the C2-SH or C2-OH in thiobestatin or bestatin, respectively, have been suggested (41, 42).

BINDING OF BESTATIN AND OTHER OUTSTANDING MYSTERIES

Bestatin has been shown to be a slow-binding, competitive inhibitor for most aminopeptidases tested (Table 1). The slow binding involves rapid formation of the initial collision complex, slow transformation of the EI complex to the tight complex EI*, and even slower deformation of that complex. It is

clear that the C2-OH in bestatin is involved in tighter binding than is observed in the analogous peptide. However, specific enzyme-inhibitor interactions that distinguish this process from interaction of aminopeptidases with substrates remain to be determined. It is curious that a delay in blLAP-catalyzed hydrolysis is observed using leucylamide as substrate (A. Taylor, unpublished results). It would be interesting to know if both "slow" events involve similar interactions in the enzyme.

There are other slow-binding inhibitors of aminopeptidases. These include boronic acids, phosphonic acids, α-methylleucine, and isoamylthioamide (reviewed in A. Taylor et al., manuscript submitted). Because of different binding kinetics, other binding mechanisms have been described for arginine aminopeptidase (43).

Several compounds that were anticipated to be inhibitors were activators of blLAP. These included Leu- and Ala-p-aminobenzenesulfonates (which are also substrates), orthanilic and sufanilic acids (16). Amastatin [(2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl-L-valyl-L-valyl-aspartic acid] is a slow binding inhibitor of aminopeptidase M. It is suggested that the tighter binding of such polypeptide inhibitors, in preference to dipeptide analogs, can be used to distinguish membrane-bound (i.e., aminopeptidase M) from cytosolic enzymes (44). A statine analog of amastatin [(3S,4S)- Sta-Val-Val-Asp] stimulated rather than inhibited LAP. The observed activation might imply the presence of a second, perhaps adjacent, binding site (45). The stability of LAP in cryosolvents should facilitate future structural and mechanistic studies (46).

Asano et al. (47) purified from *Ochrobactrum anthropi* SCRC C1-38, a dimeric (59 kDa/subunit) aminopeptidase specific for D amino acids. This enzyme is not inhibited by metal chelating agents but is inhibited by sulfhydryl reagents. High-resolution, structural information is needed to elucidate differences in design between enzymes specific for D-, as opposed to L-amino acids, the interaction of the C2-OH of bestatin and enzyme-bound metal ions and/or OH$^-$, and the surprising activity enhancement by molecules that were anticipated to be inhibitors.

HOMOLOGIES

Quantitative immunological techniques indicated that within a species LAPs are indistinguishable (48). Thus, it was not unexpected that the amino acid sequence information deduced from the bkLAP cDNA allowed solution of the blLAP structure. This also corroborated the identities of these bovine LAPs.

A search of the DNA and protein sequence data revealed sequence homologies between bovine LAP and a 55.3-kDa, 503 amino acid, xerB gene product, aminopeptidase A, from *E. coli* (24). The *E. coli* aminopeptidase A is required for ColE1 stabilization of unstable plasmid multimers, which occurs via site-specific recombination (at the cer locus) into monomer form. *E. coli* aminopeptidase A shows significant aminopeptidase activity that is activated by Mn^{2+} and is inhibited by EDTA. These properties are consistent with a zinc peptidase and are similar to that observed with bovine LAP (34). Using the amino acid sequence provided by protein sequencing, Stirling et al. (24) noted that *E. coli* aminopeptidase A has overall 31% identity of amino acids and 22% similar residues to blLAP. Identity of the protein is even greater (52%) in the COOH-terminal region. Using the deduced bkLAP amino acid sequence (B. Wallner et al., manuscript submitted), Burley et al. (25) reported 18, 44, and 35% identities for the NH$_2$-terminal and COOH-

terminal domains and for the entire protein, respectively (Fig. 2). For sequence similarity, the numbers are 46, 66, and 60%, respectively. The higher degree of homology in the COOH-terminal region may be expected, as photoaffinity-labeling experiments (40) and crystallographic (25) data indicate that the COOH-terminal region contains the active site of the bovine enzyme; thus, evolutionary constraints might be more stringent. All the residues that appear to be involved in zinc binding or catalysis in bovine LAP are conserved in *E. coli* xerB gene product, and all the residues, except Met-454 used in the inhibitor and presumably substrate binding, are also conserved. Furthermore, organized secondary structure and the loop regions comprising the active site are highly conserved between blLAP and *E. coli* aminopeptidase A (Fig. 2).

Recent data show that blLAP and *Aeromonas* LAP show 18-20% amino acid sequence identity (49). A 45% similarity of the COOH-terminal domain of blLAP and the detergent-resistant alkaline exoprotease of *Vibrio alginolyticus* was also reported (49).

Microcomplement fixation assays indicated that bovine and hog LAP share 91% sequence homology, whereas bovine and human LAP share 81% sequence homology (48). Matsushima et al. (22) noted homology between hog LAP and human liver LAP. As with blLAP and bkLAP, lens, intestine, and kidney LAPs in hog are indistinguishable. Quaternary structure similarities are indicated by similar crystallographic characteristics of the hog and bovine enzymes (28). As the enzymes are also kinetically very similar (Table 1), it is likely that they share the same active site design. Recently, it was demonstrated that prolyaminopeptidase activity is also due to the LAP enzyme (22, 23). In addition, genetic methods were used to demonstrate that the *E. coli* pepA gene is identical to the *S. typhimurium* pepA gene. The data presented here suggest that blLAP, bkLAP, human lens and liver LAPs, hlLAP, hkLAP, hog intestine LAP, prolyl aminopeptidase, *E. coli* aminopeptidase A, aminopeptidase I, and the *S. typhimurium* pepA gene product are part of a new family of zinc aminopeptidases that utilize the zinc-binding (and probably much of the substrate binding) amino acid constellations described for bovine LAP. Because the two Zn^{2+} *Aeromonas* LAP shows homology to blLAP and does not use the two His and Glu motif (see below) for metal binding, it is tempting to speculate that it may also belong to this group of enzymes. These peptidases can be distinguished from another recently identified superfamily of zinc proteases that appear to use Glu in catalysis, two His and Glu to bind zinc, and Arg in substrate binding. These include rat kidney zinc protease (rKZP), aminopeptidase N, thermolysin B.T. (*Bacillus thermoproteolyticus*), thermolysin B. S. (*Bacillus stearothermophilus*), protease B.A. (*Bacillus amyloliquefaciens*), protease *Serratia*, rat enkephalinase, carboxypeptidases A and B (50), and possibly aminopeptidases M and N, some collagenases, angiotensin-converting enzyme, human aminopeptidase M, and leukotriene A4 hydrolase. The extent of similarity between rKZP and the last two enzymes is 77 and 31%, respectively. The extent of homology between rKZP and the *E. coli* aminopeptidase N is 18% (21).

The critical functions of methionine aminopeptidases were noted earlier (Occurrence and Biological Function section). Some of these aminopeptidases have now been isolated and partially characterized in many species, and a crystal structure is forthcoming (B. Matthews, personal communication). Methionine aminopeptidase cloned from *Saccharomyces cerevisiae* is synthesized as a 387 amino acid (43 kDa) polypeptide, which is clipped to 377 residues in the mature form (51). The function of the 10 removed amino acids is

unknown. The yeast enzyme consists of two functional domains: a unique NH$_2$-terminal domain containing two motifs resembling zinc fingers, which may allow the protein to interact with ribosomes and function cotranslationally, and a catalytic COOH-terminal domain resembling other prokaryotic methionine aminopeptidases. The deduced amino acid sequence from the *Saccharomyces* methionine aminopeptidase shows approximately 40% sequence homology with methionine aminopeptidase from *E. coli*, *S. typhimurium*, and *B. subtilis*. However, they share little homology with blLAP (52). Unlike many aminopeptidases, some methionine aminopeptidases (i.e., rat liver) are not inhibited by bestatin (53). Most of the similarity between these enzymes is localized to the COOH region. This enzyme is clipped to a functional 34-kDa protein.

SUMMARY AND CONCLUSIONS

Methionine aminopeptidases are critical for the maturation of most proteins in eukaryotic and prokaryotic cells. Other aminopeptidases are essential for digestive and intracellular protein metabolism, including destabilization of—and hence regulation of levels of—hormones. By virtue of their ability to remove NH$_2$-terminal residues at differential rates, it has also recently been proposed that aminopeptidases are involved in regulation of rates of hydrolysis of proteins that are degraded by the ubiquitin-dependent pathway. Recent assessments suggest that the ubiquitin-dependent pathways are responsible for degradation of a significant amount of intracellular protein — including removal of damaged or obsolete proteins. Taken together, this indicates that aminopeptidases perform regulatory as well as "housekeeping" functions. The availability of structural and kinetic data should aid in elucidating on a molecular level the mechanism of these phenomena and should aid further studies of molecular structure, physiology, and medical uses of these enzymes. To maximize these possibilities, the structure and mechanism of more aminopeptidases must be identified. A uniform, simple nomenclature system would avoid confusion and duplication of effort. Because recent work shows that small peptides are physiological substrates for aminopeptidases in vivo and that the binding sites extend beyond the S$_1$,S$_1$' sites, it would appear that activity assays that use dipeptides or tripeptides as substrates may be more informative. Assays using LeuGlyGly, or suitable analogs in which NH$_2$-terminal peptide bond is hydrolyzed much more rapidly than the carboxyl-terminal peptide bond, may fulfill this function. [FJ]

REFERENCES

1. Linderstrom-Lang, Y. K. (1929) Uber Darmerepsin. *Hoppe-Seyler's Z. Physiol. Chem.* 182, 151–174
2. McDonald, J. K. (1986) A brief history of the study of mammalian exopeptidases. In *Mammalian Proteases* (McDonald, J. K., and Barrett, A. J., eds) Vol. 2, pp. 7–19, Academic Press, New York
3. Hanson, J. H., and Frohne, M. (1976) Crystalline leucine aminopeptidase from lens. *Methods Enzymol.* 45, 504–510
4. Delange, R. J., and Smith, E. L. (1971) Leucine aminopeptidase and other N-terminal exopeptidases. In *The Enzymes* (Boyer, P. D., ed) Vol. 3, pp. 81–103, Academic Press, New York
5. Kenny, A. J., Stephenson, S. L., and Turner, A. J. (1987) Cell surface peptidases. In *Mammalian Ectoenzymes* (Kenney, A. J., and Turner, A. J., eds) pp. 169–210, Elsevier, New York
6. Jones, E. W., Zubenko, G. S., and Parker, R. R. (1982) *Pep4* gene function is required for expression of several vacuolar hydrolases in *Saccharomyces cerevisiae*. *Genetics* 102, 665–677
7. Miller, C. G. (1975) Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. *Annu. Rev. Microbiol.* 29, 485–504
8. Barrett, A. J. (1979) In *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed) Elsevier/North-Holland Biomedical Press, Amsterdam
9. Sanderink, G. J., Artur, Y., and Siest, G. (1988) Human aminopeptidases: a review of the literature. *J. Clin. Chem. Clin. Biochem.* 26, 795–807
10. McDonald, J. K., and Barrett, A. J. (1986) In *Mammalian Proteases* (McDonald, J. K., and Barrett, A. J., eds) Vol. 2, Academic Press, New York
11. Moerschell, R. P., Hosokawa, Y., Tsunawa, S., and Sherman, F. (1990) The specificities of yeast methionine aminopeptidase and acetylation of amino-terminal methionine in vivo. *J. Biol. Chem.* 265, 19638–19643
12. Botbol, V., and Scornik, O. A. (1991) Measurement of instant rates of protein degradation in the livers of intact mice by the accumulation of bestatin-induced peptides. *J. Biol. Chem.* 266, 2151–2157
13. Bachmair, A., Finley, D., and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. *Science* 234, 179–186
14. Taylor, A., Dorey, C. K., and Jacques, P. F. (1992) Oxidation and aging: impact on vision. In *Proc. Int. Conf. on Antioxidants* (Sies, H., Erdman, J., Baker, G., Henry, C., and Williams, G., eds) Vol. 8, Princeton Scientific Press, Princeton, N.J. In press
15. Nomenclature Committee of International Union of Biochemistry (1978) *Enzyme Nomenclature*. Academic Press, New York
16. Taylor, A., Tisdell, F. E., and Carpenter, F. H. (1981) Leucine aminopeptidase (bovine lens): synthesis and kinetic properties of ortho, meta, and para substituted leucyl-anilides. *Arch. Biochem. Biophys.* 210, 90–97
17. Sanderink, G.-J., Artur, Y., and Seist, G. (1988) Human aminopeptidases: a review of the literature. *J. Clin. Chem. Clin. Biochem.* 26, 795–807
18. Lin, W.-Y., and Van Wart, H. E. (1988) Steady-state kinetics of hydrolysis of dansyl-peptide substrates by leucine aminopeptidase. *Biochemistry* 27, 5062–5068
19. Patterson, E. K. (1989) Inhibition by bestatin of a mouse ascites tumor dipeptidase. *J. Biol. Chem.* 264, 8004–8011
20. Squire, C. R., Talebian, M., Menon, J. G., Dkruyff, S., Lee, T. D., Shively, J. E., and Rothman, B. S. (1991) Leucine aminopeptidase-like activity in *Aplysia hemolynph* rapidly degrades biologically active alpha-bag cell peptide fragments. *J. Biol. Chem.* 266, 22355–22363
21. Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J.-C., and Hellmiss, R. (1989) Molecular cloning and amino acid sequence of rat kidney aminopeptidase M: a member of a super family of zinc-metallohydrolases. *Biochem. Biophys. Res. Commun.* 161, 236–241
22. Matsushima, M., Takahashi, T., Ichinose, M., Miki, K., Kurokawa, K., and Takahashi, K. (1991) Structural and immunological evidence for the identity of prolyl aminopeptidase with leucyl aminopeptidase. *Biochem. Biophys. Res. Commun.* 178, 1459–1464
23. Turzynski, A., and Mentlein, R. (1990) Prolyl aminopeptidase from rat brain and kidney. *Eur. J. Biochem.* 190, 509–515
24. Stirling, V. J., Colloms, S. D., Collins, J. F., Szatmari, G., and Sherratt, D. J. (1989) *xerB*, an *Escherichia coli* gene required for plasmid ColE1 site-specific recombination, is identical to *pepA*, encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase. *EMBO J.* 8, 1623–1627
25. Burley, S. K., David, P. R., Sweet, R. M., Taylor, A., and Lipscomb, W. N. (1992) Structure determination and refinement of

bovine lens leucine aminopeptidase and its complex with besta-tin. *J. Mol. Biol.* **224**, 113–140

26. Taylor, A., Carpenter, F. H., and Wlodawer, A. (1979) Leucine aminopeptidase (bovine lens): an electron microscopic study. *J. Ultrastruct. Res.* **68**, 92–100

27. Jurnak, F., Rich, A., van Loon-Klaassen, L., Bloemendal, H., Taylor, A., and Carpenter, F. H. (1977) Preliminary x-ray study of leucine aminopeptidase (bovine lens): an oligomeric metal-loenzyme. *J. Mol. Biol.* **112**, 149–153

28. Taylor, A., Volz, K., Lipscomb, W. N., and Takemoto, L. (1984) Leucine aminopeptidase from bovine lens and hog kidney: comparison using immunological techniques, electron microscopy and x-ray diffraction. *J. Biol. Chem.* **259**, 14757–14761

29. Cuypers, H. T., van Loon-Klaassen, L. A. H., Vree Egberts, W. T. M., de Jong, W. W., and Bloemendal, H. (1982) The primary structure of leucine aminopeptidase from bovine eye lens. *J. Biol. Chem.* **257**, 7077–7085

30. van Loon-Klaassen, L., Cuypers, H. T., and Bloemendal, H. (1979) Limited tryptic digestion of bovine eye lens leucine aminopeptidase. *FEBS Lett.* **107**, 366–370

31. Nishizawa, R., Saino, T., Takita, T., Suda, H., Aoyagi, T., and Umezawa, H. (1977) Synthesis and structure-activity relation-ships of bestatin analogues, inhibitors of aminopeptidase B. *J. Med. Chem.* **20**, 510–515

32. Wilkes, S. H., and Prescott, J. M. (1985) The slow, tight binding of bestatin and amastatin to aminopeptidases. *J. Biol. Chem.* **260**, 134154–13162

33. Schechter, I., and Berger, A. (1967) Characterization of a pyroglutamate aminopeptidase from rat serum that degrades thyrotropin-releasing hormone. *Biochem. Biophys. Res. Commun.* **27**, 157–162

34. Carpenter, F. H., and Vahl, J. M. (1973) Leucine aminopepti-dase (bovine lens): mechanism of activation by Mg^{2+} and Mn^{2+} of the zinc metalloenzyme, amino acid composition, and sulf-hydryl content. *J. Biol. Chem.* **248**, 294–304

35. Taylor, A., Sawan, S., and James, T. (1982) On the binding of eucyl-*o*-sulfonic acid in leucine aminopeptidase. Interaction be-tween this substrate analog and the activation site metal-viewed by NMR. *J. Biol. Chem.* **257**, 11571–11576

36. Bryce, G. F., and Rabin, B. R. (1964) The function of the metal ion in leucine aminopeptidase and the mechanism of action of the enzyme. *Biochem. J.* **90**, 513–518

37. Allen, M. P., Yamada, A. H., and Carpenter, F. H. (1983) Ki-netic parameters of metal-substituted leucine aminopeptidase from bovine lens. *Biochemistry* **22**, 3778–3783

38. Prescott, J. H., Wagner, F. W., Holmquist, B., and Vallee, B. L. (1985) Spectral and kinetic studies of metal-substituted *Aero-monas* aminopeptidase: nonidentical, interacting metal-binding sites. *Biochemistry* **24**, 5350–5356

39. Van Wart, H. E., and Lin, S. H. (1981) Metal binding stoichiometry and mechanism of metal ion modulation of the activity of porcine kidney leucine aminopeptidase. *Biochemistry* **20**, 5682–5689

40. Taylor, A., Peltier, C. Z., Jahngen, E. G. E., Jr., Laxman, E., and Torre, F. J. (1992) Use of azidobestatin as a photoaffinity label to identify the active site peptide of leucine aminopepti-dase. *Biochemistry* **31**, 4141–4150

41. Ocain, T. D., and Rich, D. H. (1988) Synthesis of sulfur-containing analogues of bestatin. Inhibition of aminopeptidases by alpha-thiolbestatin analogues. *J. Med. Chem.* **31**, 2193–2199

42. Gordon, E. M., Godfrey, J. D., Delaney, N. G., Asaad, M. M., von Langen, D., and Cushman, D. W. (1988) Design of novel inhibitors of aminopeptidases. Synthesis of peptide-derived di-amino thiols and sulfur replacement of analogues of bestatin. *Biochemistry* **31**, 2199–2211

43. Harbeson, S. L., and Rich, D. H. (1988) Inhibition of arginine aminopeptidase by bestatin and arphamenine analogues. Evi-dence for a new mode of binding to aminopeptidases. *Biochemis-try* **27**, 7301–7310

44. Rich, D. H., Moon, B. J., and Harbeson, S. (1984) Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding processes. *J. Med. Chem.* **27**, 417–422

45. DiGregorio, M., Pickering, D. S., and Chan, W. W.-C. (1988) Multiple sites and synergism in the binding of inhibitors to microsomal aminopeptidase. *Biochemistry* **27**, 3613–3617

46. Lin, W-Y., Lin, S. H., Morris, R., and Van Wart, H. E. (1988) Stopped-flow cryoenzymological investigation of the pre-steady-state kinetics of hydrolysis of leu-gly-NHNH-Dns by leucine aminopeptidase. *Biochemistry* **27**, 5068–5074

47. Asano, Y., Nakazawa, A., Kato, Y., and Kondo, K. (1989) Properties of a novel D-stereospecific aminopeptidase from *Ochrobactrum anthropi.* *J. Biol. Chem.* **264**, 14233–14239

48. Taylor, A., Surgenor, T., Thomson, D. K. R., Graham, R. J., and Oettgen, H. C. (1984) Comparison of (concentration and amino acid sequence homology between) leucine aminopepti-dase in human lens, beef lens, beef kidney, hog lens, and hog kidney. *Exp. Eye Res.* **38**, 217–229

49. Guenet, C., Lepage, P., and Harris, B. A. (1992) Isolation of the leucine aminopeptidase gene from *Aeromonas proteolytica.* *J. Biol. Chem.* **267**, 8390–8395

50. Watt, V. M., and Yip, C. C. (1989) Amino acid sequence deduced from a rat kidney cDNA suggests it encodes the Zn-peptidase aminopeptidase N. *J. Biol. Chem.* **264**, 5480–5487

51. Chang, Y.-H., Teichert, U., and Smith, J. A. (1992) Molecular cloning, sequencing, deletion, and overexpression of a methio-nine aminopeptidase gene from *Saccharomyces cerevisiae.* *J. Biol. Chem.* **267**, 8007–8011

52. Ben-Bassat, A., Bauer, K., Sheng-Yung, C., Myambo, K., Boos-man, A., and Chang, S. (1987) Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. *J. Bacteriol.* **169**, 751–757

53. Freitas, J. O., Termignoni, C., and Guimaraes, J. A. (1985) Microsomal methionine aminopeptidase: properties of the de-tergent solubilized enzyme. *Int. J. Biochem.* **17**, 1285–1291

54. Suda, H., Aogi, T., Takeuchi, T., and Umezawa, H. (1976) Inhi-bition of aminopeptidase B and leucine aminopeptidase by bestatin and its stereoisomer aminopeptidase: nonidentical, in-teracting metal-binding sites. *Arch. Biochem. Biophys.* **177**, 196–200

55. Nishimura, T., Rhyu, M. R., and Kato, H. (1991) Purification and properties of aminopeptidase N from porcine skeletal mus-cle. *Agric. Biol. Chem.* **55**, 1779–1786

56. Cueva, R., Garcia-Alvarez, N., and Suarez-Rendueles, P. (1989) Yeast vacuolar aminopeptidase yscI isolation and regula-tion of the *APE1 (LAP4)* structural gene. *FEBS Lett.* **259**, 125–129

57. Chang, Y.-H., and Smith, J. A. (1989) Molecular cloning and sequencing of genomic DNA encoding aminopeptidase I from *Saccharomyces cerevisiae.* *J. Biol. Chem.* **264**, 6979–6983

58. Woolford, C. A., Daniels, L. B., Park, F. J., Jones, E. W., Van Arsdell, J. N., and Innis, M. A. (1986) The *pep4* gene encodes an aspartyl protease implicated in the posttranslational regula-tion of *Saccharomyces cerevisiae* vacuolar hydrolases. *Mol. Cell. Biol.* **6**, 2500–2510

59. Miller, C. G., Strauch, K. L., Kukral, A. M., Miller, J. L., Wingfield, P. T., Mazzei, G. J., Werlen, R. C., Graber, P., and Movva, N. R. (1987) N-terminal methionine-specific peptidase in *Salmonella typhimurium.* *Proc. Natl. Acad. Sci. USA* **84**, 2718–2722